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Herpes simplex virus 2 ICP34.5 confers neurovirulence by regulating the type I interferon response

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ABSTRACT

The γ34.5 gene of herpes simplex virus (HSV) 2 encodes ICP34.5, which enhances HSV-2 neurovirulence by an unknown mechanism. We found that an HSV-2 γ34.5-null mutant ($\gamma34.5^{-/-}$) replicated less robustly than its rescue virus (γ34.5R) in wild-type mouse embryo fibroblasts (MEFs), and in cells primed with IFNβ. Increased eIF2α phosphorylation correlated with $\gamma34.5^{-/-}$ attenuation. However, $\gamma34.5^{-/-}$ achieved titers equivalent to γ34.5R in MEFs lacking the type I IFN receptor ($IFN\alpha/\beta R^{-/-}$) or lacking protein kinase R. $\gamma34.5^{-/-}$ also replicated poorly in the vaginal mucosa of wild-type mice, caused little genital inflammation, and spread to the nervous system at lower levels compared to γ34.5R. In $IFN\alpha/\beta R^{-/-}$ mice, however, $\gamma34.5^{-/-}$ regained the capacity to replicate and cause disease equivalent to γ34.5R after intravaginal infection or direct inoculation into the central nervous system. Thus, the capacity of HSV-2 ICP34.5 to interdict the type I IFN response in vivo largely determines its neurovirulence.

Introduction

Herpes simplex viruses (HSVs) are neurotropic, and after initial infection of an epithelial surface virus spreads to the cell body of sensory neurons innervating the epithelium. HSV-1 typically infects oral or corneal epithelia whereas HSV-2 causes most genital ulcerative disease. A hallmark of HSV infection is maintenance of the viral genome in a repressed, latent state in sensory nerve ganglia. Reactivation of the viral genome to a state of productive replication results in asymptomatic viral shedding from the epithelium or recurrent ulcerative disease. The capacity to enter latency prevents HSV from being cleared and this results in a high global burden of infection; an estimated 500 million people are infected with HSV-2 (Looker et al., 2008). When virus ascends from peripheral neurons to the CNS, replication can lead to relatively rare but severe neurological disease. This is particularly evident in immunocompromised patients (Mommeja-Marin et al., 2003; Jancel and Penzak, 2009) and in infants infected during birth (Kimberlin and Whitley, 2005; Pinninti and Kimberlin, 2013).

The highly related genomes of HSV-1 and HSV-2 contain two copies of the γ34.5 gene located in the inverted repeats (McGeoch et al., 1991). The γ34.5 gene product, infected cell protein (ICP) 34.5, contributes significantly to HSV pathogenesis and also to neurovirulence, as defined by attenuation after intracranial (i.c.) inoculation into mice of virus containing a deletion or mutation in γ34.5 (Taha et al., 1989a, 1989b; Chou et al., 1990; Bolovan et al., 1994; Valyi-Nagy et al., 1994). The role of γ34.5 in HSV-1 pathogenesis has been linked to its capacity to counteract the type I IFN response. For example, following ocular infection an HSV-1 γ34.5-null mutant replicates to a greater extent in the cornea and trigeminal ganglia of mice that lack the common type I IFN receptor ($IFN\alpha/\beta R^{-/-}$) than it does in wild-type (wt) mice (Leib et al., 1999). An HSV-1 γ34.5-null mutant also regains the virulence of wt virus after i.c. inoculation into $IFN\alpha/\beta R^{-/-}$ mice (Leib et al., 2000).

The mechanisms by which HSV-1 ICP34.5 counteracts the type I IFN response have been well-characterized. HSV-1 ICP34.5 binds four cellular proteins: Beclin-1 (Orvedahl et al., 2007) and tank-binding kinase 1 (TBK1) (Verpooten et al., 2009) via its N-terminal domain, and protein phosphatase 1α (PP1α) (He et al., 1998; Cheng et al., 2001b) and eukaryotic translation initiation factor 2α (eIF2α) (Li et al., 2011) via its C-terminal domain. HSV-1 ICP34.5 binding of TBK1 inhibits the induction of IFNβ in transfection experiments (Verpooten et al., 2009; Ma et al., 2012), and a virus with an N-terminal deletion that includes the TBK1 binding domain replicates very poorly in the cornea and does not reach the nervous system (Ma et al., 2012). PP1α dephosphorylates eIF2α when binding by ICP34.5 brings them into close proximity (He et al., 1998; Li et al., 2011), thus counteracting IFN-stimulated protein kinase R (PKR) activity to prevent translational shutoff (He et al., 1997, 1998). Amino acid residues within the PP1α binding domain are critical determinants of HSV-1 IFN resistance (Cheng et al.,

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2001a), and a virus with mutation of these residues similarly is highly attenuated when inoculated onto the cornea (Verpooten et al., 2009). Lastly, HSV-1 ICP34.5 binds and sequesters the pro-autophagic mediator Beclin-1 to prevent the formation of autophagosomes (Orvedahl et al., 2007) in a process also dependent on PKR-mediated phosphorylation of eIF2α (Talloczy et al., 2002). The binding of HSV-1 ICP34.5 to Beclin-1 (Orvedahl et al., 2007), and antagonism of the PKR signaling pathway (Leib et al., 2000; Orvedahl et al., 2007) contribute to neurovirulence.

An HSV-2 mutant with deletions of γ34.5 and a large amount of upstream sequence was used to identify HSV-2 ICP34.5 as a major neurovirulence factor (Taha et al., 1989a, 1989b). The severity of HSV-2 infections also depends in part on virus control of the type I IFN response (Murphy et al., 2003; Gill et al., 2006). Some evidence suggests the analogous eIF2α and PP1α binding domains of HSV-2 ICP34.5 are functional (Wylie et al., 2009; Tang et al., 2013), and indeed, strong sequence conservation with HSV-1 ICP34.5 in the C-terminal domain supports this contention. However, the precise role of HSV-2 ICP34.5 in neurovirulence, the effect of HSV-2 ICP34.5 on the type I IFN response, and whether control of the IFN response by ICP34.5 contributes to HSV-2 neurovirulence have not been established. Therefore, we investigated whether HSV-2 ICP34.5 counteracts the type I IFN response, and the potential impact of IFN resistance on ICP34.5's role in HSV-2 neurovirulence.

Results

Construction of HSV-2 γ34.5-null mutant and rescue viruses

The HSV-2 isolate used to identify γ34.5 as a neurovirulence factor (variant JH2604) contains a lesion that deleted the γ34.5 open reading frame (ORF) and more than 500 bp of the 5′ untranslated region (Taha et al., 1989a, 1989b). In order to examine the precise function of γ34.5 in HSV-2 replication and virulence, we created a γ34.5 null mutant ($\gamma34.5^{-/-}$) in the HSV-2 strain 333 background (333 wt) (Fig. 1A) which specifically targets γ34.5 to prevent ICP34.5 expression while preserving other features of this genomic region (Tang et al., 2008, 2009; Jurak et al., 2010). Two mutations were made in the γ34.5 ORF: a stop codon was inserted at amino acid 13, and a M35A mutation was introduced to alter the only internal methionine (Fig. 1A). A plasmid expressing the mutated γ34.5 ORF was co-transfected with full-length wt viral DNA and isolated plaques were screened by PCR. A recombinant virus in which of both copies of γ34.5 had been replaced was plaque-picked to homogeneity to generate $\gamma34.5^{-/-}$. To ensure that only the intended sites were altered in $\gamma34.5^{-/-}$, it was rescued back to the wt sequence by homologous recombination and the resultant virus was termed γ34.5R. Western blot of Vero cell lysates using rabbit antiserum revealed two forms of HSV-2 ICP34.5 in lysates prepared from γ34.5R- and 333 wt-infected cells (Fig. 1B), consistent with previous observations (Tang et al., 2008; Wylie et al., 2009; Korom et al., 2014). ICP34.5 was not detected in lysates from uninfected or $\gamma34.5^{-/-}$-infected samples (Fig. 1B).

To examine whether the mutations in the γ34.5 ORF altered virus replication, growth over time post-infection was determined in Vero cells infected at low multiplicity of infection (MOI). Vero cells are highly permissive to HSV infection because of deletions in the alpha- (α) and beta1- (β1) IFN genes (Emeny and Morgan, 1979; Mosca and Pitha, 1986). Infection with $\gamma34.5^{-/-}$, γ34.5R, and 333 wt viruses at MOI of 0.01 pfu/cell yielded overlapping growth curves (Fig. 1C), demonstrating $\gamma34.5^{-/-}$ has no intrinsic replication defect. Infection at high MOI (10 pfu/cell) also resulted in similar titers over time (data not shown). To rigorously verify that γ34.5R was rescued to 333 wt phenotype, groups of 129 mice were infected intravaginally (i.vag.) with γ34.5R or 333 wt. Replication in the vaginal mucosa over time post-infection was highly similar between the two viruses (Fig. 1D), as was viral titer in the CNS at 6 d post-infection (d p.i.) (Fig. 1E). We therefore compared $\gamma34.5^{-/-}$ with γ34.5R in subsequent experiments because γ34.5R phenotypically and genotypically rescued $\gamma34.5^{-/-}$ back to 333 wt.

The type I IFN response attenuates HSV-2 $\gamma34.5^{-/-}$ replication in MEFs

To determine whether HSV-2 ICP34.5 contributes to type I IFN resistance, we first asked whether replication of HSV-2 lacking ICP34.5 is attenuated in a type I IFN sufficient environment. MEFs isolated from 129 wt mice and IFNα/β$R^{-/-}$ mice were infected at low MOI with either HSV-1 Δγ34.5 and its rescue virus, Δγ34.5R, as positive control for IFNα/β sensitivity, or with HSV-2 $\gamma34.5^{-/-}$ and γ34.5R. In wt MEFs, Δγ34.5 replicated at much lower levels than Δγ34.5R from 24 to 48 h p.i. (Fig. 2A). The HSV-2 mutant followed the same pattern in that $\gamma34.5^{-/-}$ replication was significantly reduced compared with γ34.5R from 24 to 48 h p.i. (Fig. 2B). In MEFs unable to signal through the type I IFN receptor, HSV-1 Δγ34.5 replication recovered to within 40-fold of Δγ34.5R (Fig. 2C), indicating a role for type I IFN in its attenuation. Strikingly, replication of the HSV-2 $\gamma34.5^{-/-}$ mutant fully recovered to the level of γ34.5R in IFNα/β$R^{-/-}$ MEFs (Fig. 2*D*). The HSV-2 $\gamma34.5^{-/-}$ mutant also replicated like γ34.5R in both wt and IFNα/β$R^{-/-}$ MEFs after infection at high MOI (data not shown), where autocrine production of type I IFN is insufficient to inhibit ongoing HSV infection (Duerst and Morrison, 2004).

Effect of HSV-2 ICP34.5 on IFNβ priming

High titers of HSV-2 $\gamma34.5^{-/-}$ in IFNα/β$R^{-/-}$ MEFs (Fig. 2) suggested HSV-2 ICP34.5 participates in inhibition of the type I IFN response. To determine whether HSV-2 γ34.5 can overcome priming by type I IFN, wt MEFs were pretreated with 0 or 100 IU/ml of IFNβ and then infected with HSV-2 $\gamma34.5^{-/-}$ or γ34.5R at MOI of 10. Both viruses replicated to high titers by 24 h p.i. in untreated MEFs (Fig. 3A). In MEFs pretreated with IFNβ, γ34.5R still replicated efficiently but replication of $\gamma34.5^{-/-}$ was significantly reduced. We also pretreated primary human foreskin fibroblasts with 0 or 100 IU/ml of IFNβ, and again γ34.5R replicated efficiently under both conditions (Fig. 3B), but $\gamma34.5^{-/-}$ replication was attenuated. Similarly, HSV-1 Δγ34.5 replication was suppressed to a greater extent than Δγ34.5R in IFN-pretreated cells (data not shown). Levels of suppression in HFFs pretreated with 1000 IU/ml of IFNβ were similar to pretreatment with 100 IU of IFNβ (data not shown).

Type I IFN signaling triggers PKR induction and subsequent infection activates PKR to phosphorylate eIF2α, causing translational arrest. eIF2α phosphorylation positively correlates with attenuation of an HSV-2 mutant expressing reduced amounts of ICP34.5 (Wylie et al., 2009), and HSV-2 γ34.5 transfection alleviates eIF2α phosphorylation in cells infected with γ34.5-deficient HSV-1 (Tang et al., 2013). We therefore used $\gamma34.5^{-/-}$ to determine whether HSV-2 ICP34.5 opposes PKR-mediated eIF2α phosphorylation during HSV-2 infection. As expected, HSV-1 Δγ34.5R and HSV-2 γ34.5R suppressed phosphorylated eIF2α below the level in mock-infected MEFs (Fig. 3C). In contrast, more phosphorylated eIF2α accumulated in MEFs infected with Δγ34.5 or $\gamma34.5^{-/-}$ compared to mock-infected cells and cells infected with the wild-type viruses (Fig. 3C). $\gamma34.5^{-/-}$ infection of HFFs yielded similar results (data not shown). These observations provide evidence that HSV-2 $\gamma34.5^{-/-}$ cannot counteract the PKR pathway. Consistent with this interpretation, $\gamma34.5^{-/-}$ replicated as

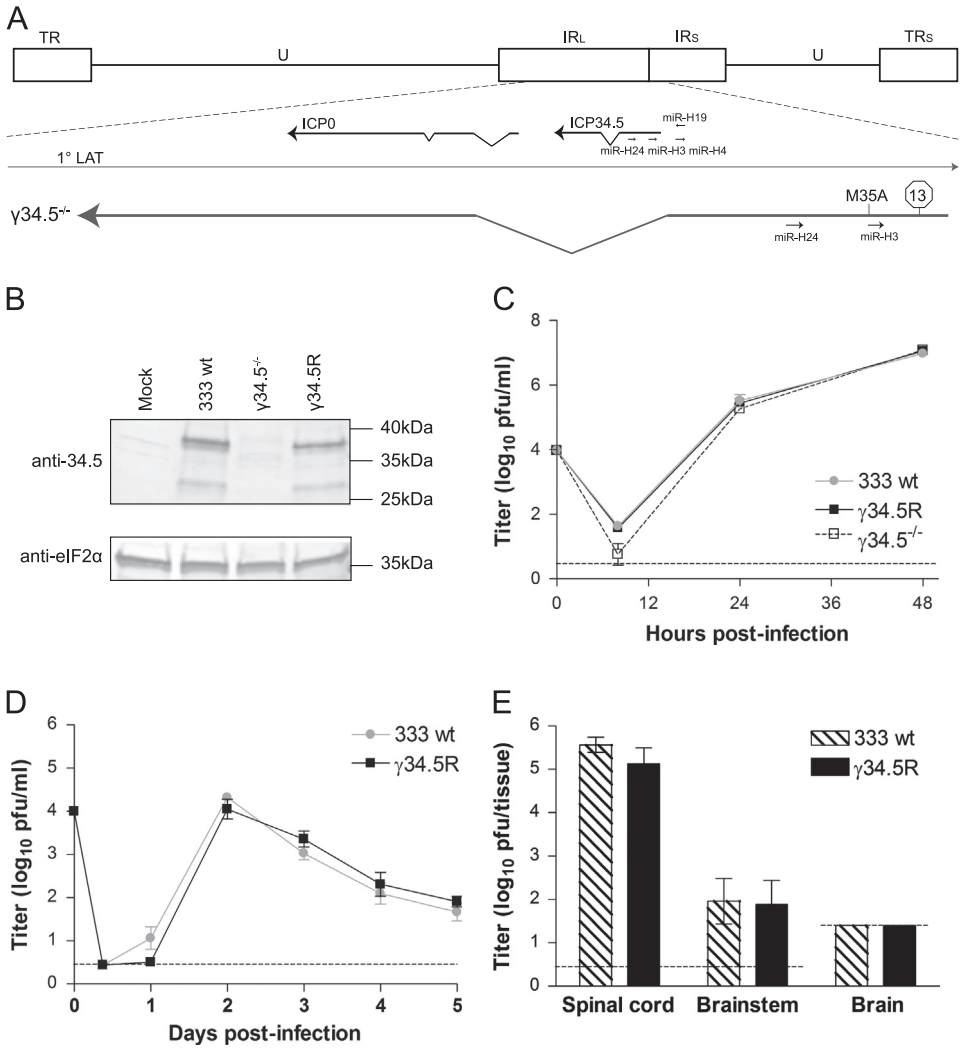

Fig. 1. Construction of HSV-2 γ34.5 null mutant and rescue viruses. (A) The genomic structure of HSV-2 γ34.5 (line 1) and γ34.5 mutagenesis scheme. An expanded view of the long inverted repeat (line 2) shows the location and orientation of transcripts encoding ICP0 and ICP34.5, and miRs within the γ34.5 locus. Line 3 shows the γ34.5 ORF. Line 4 shows mutagenesis to insert a stop codon at amino acid 13 and an M35A mutation in the γ34.5 ORF. (B) Western blot of HSV-2 ICP34.5. Vero cells were mock infected or infected at MOI of 5 with HSV-2 333 wt, γ34.5$^{-/-}$, or γ34.5R and harvested 12 h p.i. The blot was probed with rabbit polyclonal antiserum to ICP34.5 or to total eIF2α. (C) Replication of the viruses after low MOI (0.01 pfu/cell) infection of Vero cells. Cultures were collected at the indicated time points and viral titers were determined by plaque assay. Data represent the geometric mean ± SEM of triplicate cultures from one representative experiment out of two performed. (D) Titer of virus shed from the vaginal mucosa of wt mice infected i.vag. with 1×10^4 pfu/mouse of the indicated virus. (E) Viral titer in nervous system tissues, determined 6 d p.i. Data in D and E represent the geometric mean+SEM of a total of 7 to 8 mice per group compiled from two independent experiments. Dotted lines in panels C–E represent limit of detection in the plaque assay, which is higher for brain due to disruptive effects of the dense tissue homogenate on the plaque assay cell monolayer at high concentration.

well as γ34.5R in PKR$^{-/-}$ MEFs, even when the cells had been pretreated with IFNβ (Fig. 3D).

Replication and disease induced by γ34.5$^{-/-}$ after vaginal infection of mice

To determine whether HSV-2 ICP34.5 also counteracts the type I IFN response in vivo, 129 wt and IFNα/βR$^{-/-}$ mice were infected i.vag. with HSV-2 γ34.5$^{-/-}$ or γ34.5R. In wt mice, γ34.5$^{-/-}$ and γ34.5R replicated to similar extents in the vaginal mucosa through the first 3 d p.i. (Fig. 4A). γ34.5$^{-/-}$ titers rapidly dropped thereafter, whereas replication of γ34.5R continued unabated. Genital inflammation progressed quickly to lesion formation by 6 d p.i. in many wt mice infected with γ34.5R (Fig. 4B). In contrast, γ34.5$^{-/-}$ infection caused significantly less inflammation and no lesions were observed. High titers of γ34.5R were found in the spinal cord and brainstem at 6 d p.i. (Fig. 4C). Significantly lower titers of γ34.5$^{-/-}$ were recovered from the spinal cord, and virus was below the level of detection in the brain and brainstem (Fig. 4C). Thus by all measures, HSV-2 γ34.5$^{-/-}$ infection in wt mice was attenuated compared with γ34.5R. In contrast, γ34.5$^{-/-}$ and γ34.5R both achieved high titers in the vaginal mucosa of IFNα/βR$^{-/-}$ mice by 2 d p.i. and both viruses continued to replicate efficiently through 5 d p.i. (Fig. 4D). Severe genital inflammation was observed in IFNα/βR$^{-/-}$ mice infected with either γ34.5$^{-/-}$ or γ34.5R, and lesions developed by day 6 (Fig. 4E). In the spinal cords and brainstems of IFNα/βR$^{-/-}$ mice, γ34.5$^{-/-}$ and γ34.5R replicated to comparable titers, and titers in the brain were not significantly different (Fig. 3F). Thus, the attenuation of γ34.5$^{-/-}$ replication and capacity to cause disease relative to γ34.5R was abolished in IFNα/βR$^{-/-}$ mice.

Limited genital disease and low CNS titers in wt mice on day 6 after γ34.5$^{-/-}$ infection (Fig. 4A) could possibly have been due to delayed kinetics of viral dissemination from the vaginal mucosa. To determine whether γ34.5$^{-/-}$ infection eventually became as severe as γ34.5R, mice were infected i.vag. with γ34.5$^{-/-}$ or γ34.5R and monitored daily for morbidity. All wt mice infected with γ34.5R succumbed to disease by 9 d p.i., while 40% of wt mice

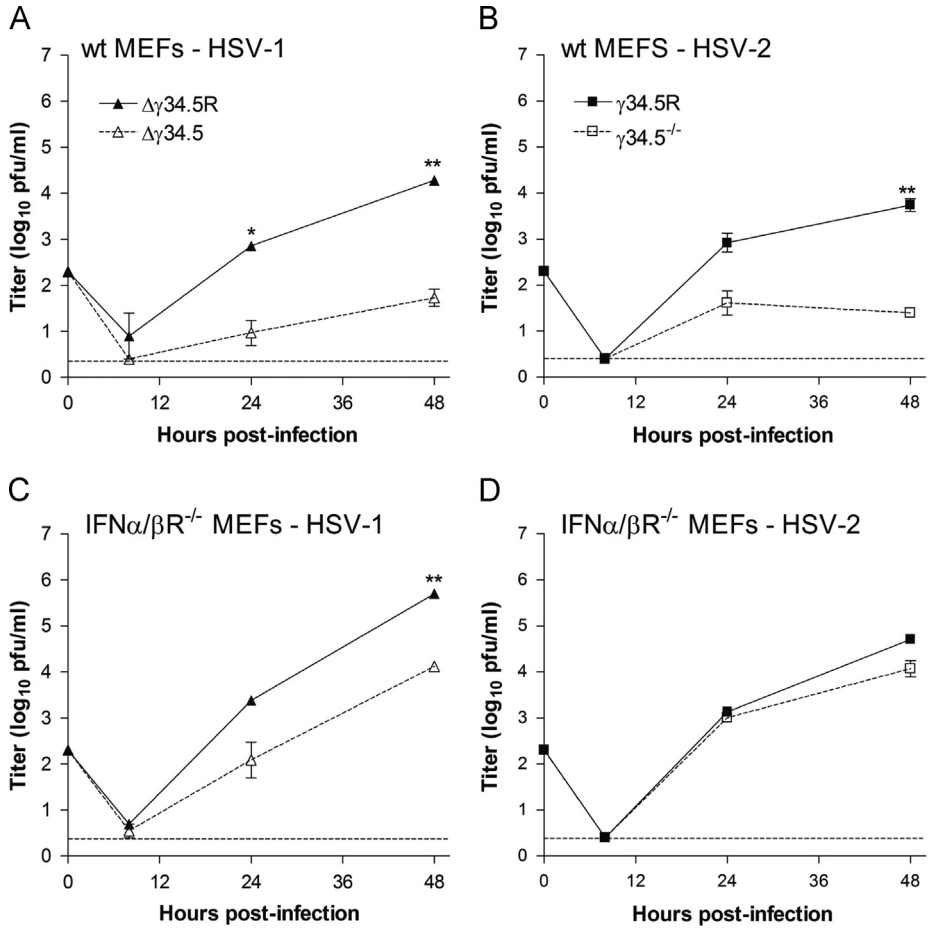

Fig. 2. $\gamma34.5^{-/-}$ replication recovers to the level of γ34.5R in IFNα/β$R^{-/-}$ MEFs. (A and B) Wt MEFs and (C and D) IFNα/β$R^{-/-}$ MEFs were infected with (A,C) HSV-1 and (B,D) HSV-2 strains at low MOI. Cultures were collected at the indicated time points and viral titers were determined by plaque assay. Data represent the geometric mean ± SEM of duplicate cultures from one representative experiment of two performed. *, $p=0.05–0.01$; **, $p=0.0099–0.001$. Dotted line represents limit of detection in the plaque assay.

infected with $\gamma34.5^{-/-}$ survived the infection (Fig. 5A). In contrast, all IFNα/β$R^{-/-}$ mice infected with either HSV-2 $\gamma34.5^{-/-}$ or γ34.5R died by 8 d p.i. (Fig. 5B).

$\gamma34.5^{-/-}$ replication after intracranial inoculation

Limited replication of $\gamma34.5^{-/-}$ in the CNS of wt mice after i. vag. infection could have resulted from reduced dissemination of virus into the CNS from the vaginal mucosa rather than relative inability to replicate in neural tissues. To directly assess the capacity of $\gamma34.5^{-/-}$ to replicate in the CNS, wt and IFNα/β$R^{-/-}$ mice were infected i.c. with $\gamma34.5^{-/-}$ and γ34.5R. 24 h p.i., γ34.5R titers in the brain had increased 45-fold over the inoculum dose (Fig. 6A). However, the amount of $\gamma34.5^{-/-}$ virus recovered was lower than the inoculum, indicating $\gamma34.5^{-/-}$ did not replicate efficiently in the brain. Correspondingly, wt mice infected i.c. with γ34.5R precipitously succumbed to infection, but survival of mice infected with $\gamma34.5^{-/-}$ was much more protracted and 30% of the mice ultimately recovered (Fig. 6B). In IFNα/β$R^{-/-}$ mice, high titers of both $\gamma34.5^{-/-}$ and γ34.5R were recovered 24 h p.i. (Fig. 6C). To ensure that recovery of mutant virus replication in the absence of a type I IFN response was not a general phenomenon, we also tested an HSV-2 thymidine kinase (TK) mutant, ΔTK^{-}. Like $\gamma34.5^{-/-}$, ΔTK^{-} replicated inefficiently after direct injection into the CNS of wt mice (Fig. 6A). However, in contrast to $\gamma34.5^{-/-}$, replication of ΔTK^{-} did not recover in IFNα/β$R^{-/-}$ mice (Fig. 6C). All IFNα/β$R^{-/-}$ mice died by 5 d p.i. whether they had been infected with $\gamma34.5^{-/-}$ or γ34.5R (Fig. 6D). Therefore, ICP34.5 specifically controls HSV-2 neurovirulence through its capacity to counteract the type I IFN response.

Discussion

HSV-2 ICP34.5 was implicated long ago as an important neurovirulence factor. An HSV-2 with a targeted mutation that disrupts only ICP34.5 expression is attenuated for replication relative to its rescue virus in wt MEFs and mice, but recovers to the level of γ34.5R in IFNα/β$R^{-/-}$ MEFs and mice. Similarly, disease progression after peripheral inoculation of $\gamma34.5^{-/-}$ resembles γ34.5R in IFNα/β$R^{-/-}$ but not wt mice. These observations indicate ICP34.5 contributes to HSV-2 pathogenesis in vivo through a mechanism dependent on inhibition of the type I IFN response. $\gamma34.5^{-/-}$ is attenuated compared with γ34.5R after i.c. inoculation of wt mice, demonstrating that HSV-2 ICP34.5 is indeed a major neurovirulence factor. In contrast, $\gamma34.5^{-/-}$ replicates efficiently in the brain of IFNα/β$R^{-/-}$ mice and is highly lethal after i.c. inoculation. Thus, the capacity of ICP34.5 to interdict the type I IFN response in vivo is critical to its role in neurovirulence.

We found that HSV-2 ICP34.5 specifically overcomes IFNβ priming in mouse and human fibroblasts. γ34.5R replication was reduced only 5-fold in IFNβ-pretreated MEFs and HFFs, whereas $\gamma34.5^{-/-}$ replication was attenuated more than 250-fold, indicating a critical role for ICP34.5 in counteracting signals transmitted through the IFNα/βR. Type I IFN signaling primes a cell for

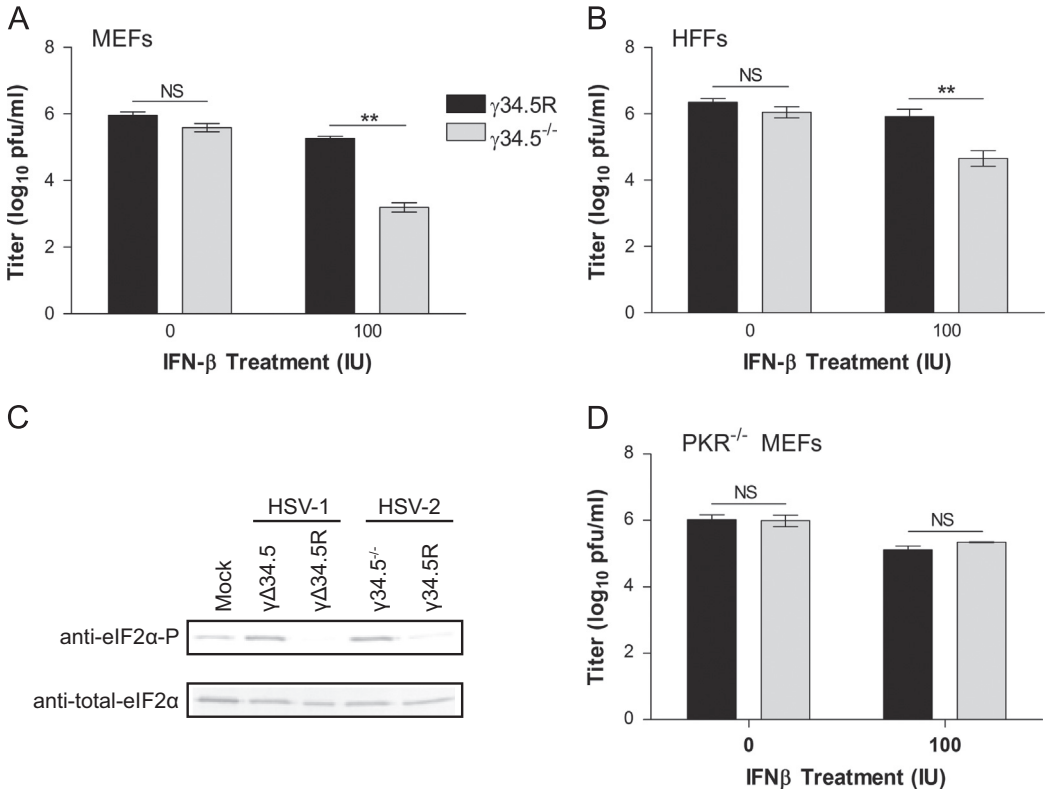

Fig. 3. ICP34.5 helps HSV-2 overcome IFNβ priming and eIF2α phosphorylation. (A) Wt MEFs and (B) HFFs were pretreated with 100 IU/ml of species-specific IFNβ or with medium only. 24 h after addition of IFNβ, cells were infected at high MOI (10 pfu/cell) with the indicated viruses. Well contents were collected 24 h p.i. and viral titers were determined by plaque assay. Data represent the geometric mean ± SEM of duplicate cultures from one representative experiment of two performed. **, $p=0.009–0.001$. (C) Wt MEFs were infected at high MOI with the indicated viruses and cells were lysed 10 h p.i. Accumulation of phosphorylated and total eIF2α was analyzed by western blot. (D) PKR$^{-/-}$ MEFs were pretreated with 0 or 100 IU/ml of IFNβ and subsequently infected as in (A) and (B). Viral titers were determined on samples collected 24 h p.i.

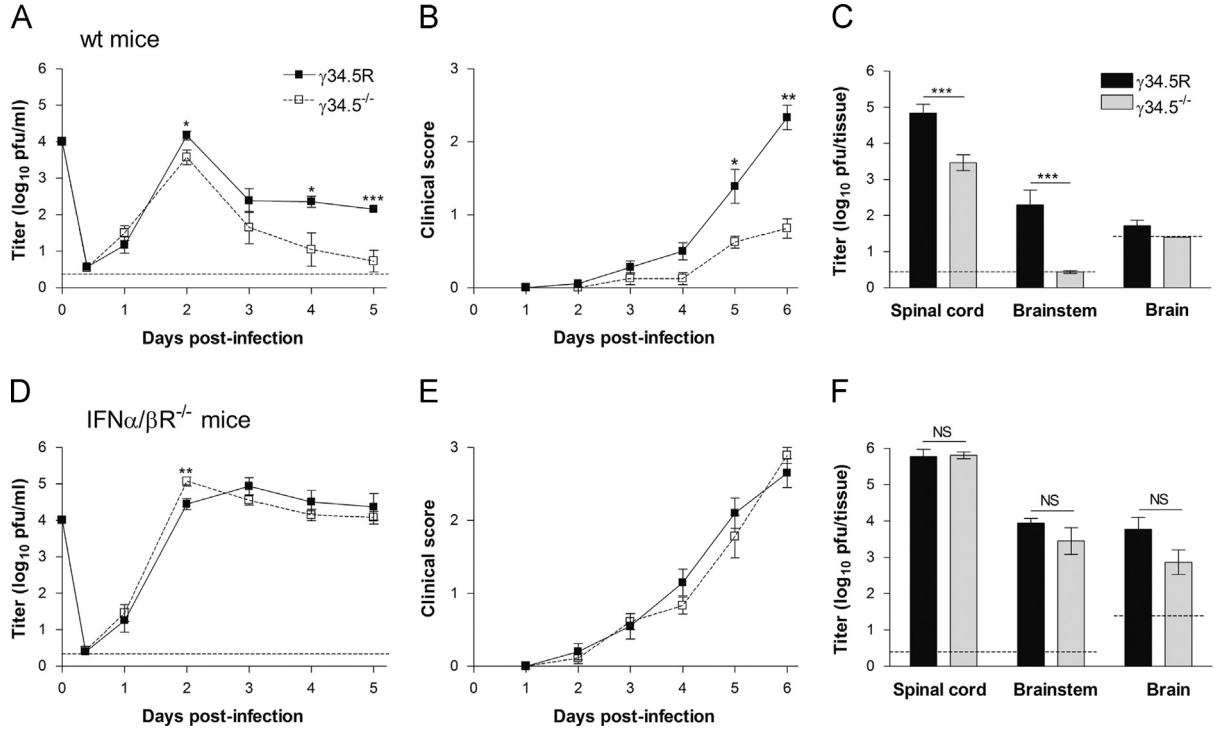

Fig. 4. Replication and disease induced by γ34.5$^{-/-}$ resemble γ34.5 R in IFNα/βR$^{-/-}$ mice. (A–C) Wt mice and (D–F) IFNα/βR$^{-/-}$ mice were infected i.vag. with 1×10^4 pfu/mouse of the indicated virus. (A,D) Vaginal swabs and (B,E) disease scores were obtained daily. The titer of virus on swabs was determined by plaque assay. (C,F) Viral titer in nervous system tissues was determined 6 d p.i. Data represent the geometric mean ± SEM of a total of 10 mice per group compiled from three independent experiments. *, $p=0.05–0.01$; **, $p=0.009–0.001$; ***, $p<0.0001$. Dotted line represents limit of detection in the plaque assay.

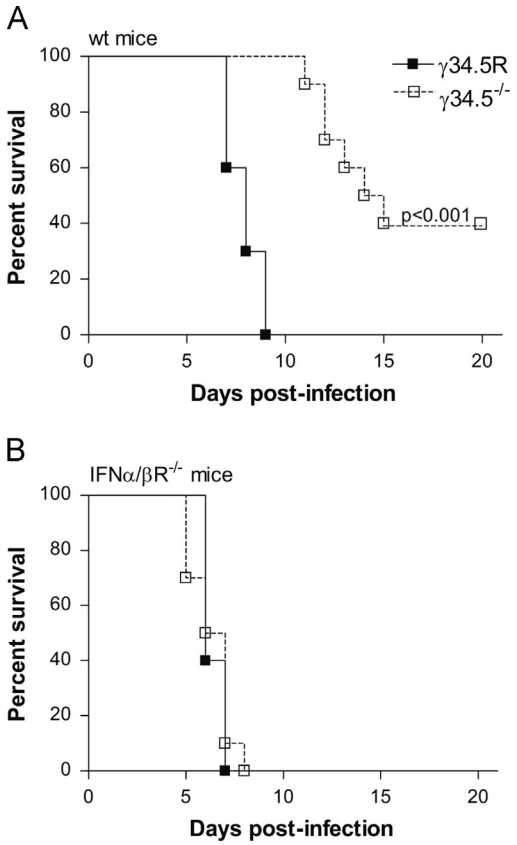

Fig. 5. $\gamma34.5^{-/-}$ is highly lethal after i.vag. infection of IFNα/β$R^{-/-}$ mice. (A) Wt mice and (B) IFNα/β$R^{-/-}$ mice were infected i.vag. as in Fig. 3 and survival was monitored daily. Data represent the day of death of a total of 10 mice per group compiled from three independent experiments.

translational shutoff in response to virus infection by activating kinases to phosphorylate eIF2α. HSV-1 ICP34.5 opposes this antiviral response by bringing PP1α and eIF2α into apposition, leading to eIF2α dephosphorylation and uninterrupted translation. The eIF2α and PP1α binding domains in HSV-1 ICP34.5 are highly conserved in HSV-2, and we had previously found evidence of functional conservation in that HSV-2 ICP34.5 protein levels inversely correlate with eIF2α phosphorylation during HSV-2 infection (Wylie et al., 2009). In addition, expression of HSV-2 ICP34.5 by transient transfection was shown to prevent hyperphosphorylation of eIF2α in cells infected with an HSV-1 γ34.5 mutant (Tang et al., 2013). Here, we formally demonstrate that HSV-2 lacking ICP34.5 cannot oppose eIF2α phosphorylation. Cells exposed to IFNβ upregulate kinases responsible for eIF2α phosphorylation, making them inhospitable to virus replication. Restoration of $\gamma34.5^{-/-}$ replication in IFNβ-treated, PKR-deficient MEFs indicates a principal role for PKR this antiviral response to HSV-2 infection. Thus, HSV-2 ICP34.5 binding of eIF2α and PP1α may provide the mechanism underlying HSV-2 ICP34.5's contribution to neurovirulence.

Our results that HSV-2 ICP34.5 disrupts the type I IFN response by opposing post-receptor signaling inform our observation that $\gamma34.5^{-/-}$ replication recovered to the level of γ34.5R in MEFs infected at low MOI. Under conditions of a low MOI infection, the few infected cells in a monolayer or region of tissue are induced to release type I IFNs, which then have a paracrine effect on neighboring uninfected cells, signaling through the IFNα/βR to prime for an antiviral response. The attenuation of HSV-2 $\gamma34.5^{-/-}$ compared with γ34.5R in wt MEFs infected at low MOI demonstrates the effectiveness of this antiviral response in the absence of ICP34.5 inhibition. IFNα/β$R^{-/-}$ MEFs which are incapable of sensing type I IFNs permit $\gamma34.5^{-/-}$ to replicate unabated. These results indicate ICP34.5 critically influences the replicative capacity of HSV-2 early during infection when the virus faces a rapid innate immune response. Interestingly, titers of HSV-2 $\gamma34.5^{-/-}$ recovered to a greater extent than HSV-1 Δγ34.5 in IFNα/β$R^{-/-}$ MEFs (Fig. 2C and D), even though HSV-1 ICP34.5 can inhibit both IFN induction and responsiveness (Cheng et al., 2001a; Verpooten, Ma et al., 2009), and replication of a γ34.5-deleted HSV-1 is completely restored in $PKR^{-/-}$ MEFs (Cheng et al., 2001b). These observations suggest another antiviral pathway independent of type I IFN signaling but dependent on PKR contributes to suppression of HSV-1 lacking ICP34.5. HSV-1 ICP34.5 binds Beclin-1 to help the virus oppose PKR-dependent autophagy (Talloczy et al., 2002; Orvedahl et al., 2007), and the pro-autophagic antiviral effects of the prion protein, PrP (Korom et al., 2013). It will be interesting to determine whether HSV-1 and HSV-2 ICP34.5 differ in their capacity to bind Beclin-1 and counteract PrP function, as the low amino acid sequence conservation in the Beclin-1 binding domain would suggest. Alternatively, HSV-1 Δγ34.5 may remain partially attenuated in IFNα/β$R^{-/-}$ MEFs because lack of ICP34.5 reduces expression the late gene Us11 (Mulvey et al., 2003) which also helps preclude translational shutoff (Mulvey et al., 1999). HSV-2 Us11 expression may therefore be independent of ICP34.5 or may not have the same influence on virus fitness.

Type I IFN plays an important role in limiting HSV-2 replication in the mucosa and development of disease. From its peak at 2 d p.i., replication of $\gamma34.5^{-/-}$ virus is rapidly controlled (Fig. 4A), demonstrating the impact of the type I IFN response on a virus that cannot counteract it. Rescue virus replication in the vaginal mucosa of wt mice also diminishes after 2 d p.i.; however, high titers persist in IFNα/β$R^{-/-}$ mice, as we have previously observed (Murphy et al., 2003; Korom et al., 2008). Thus, even wild-type HSV-2 cannot completely overcome the effects of type I IFN in vivo. Two major factors contribute to genital inflammation: damage due to lytic virus replication (Parr et al., 1994), and the activities of immune cells that infiltrate the infected mucosa (Carr and Tomanek, 2006; Duerst and Morrison, 2007). The control of HSV-2 infection in the periphery exerted by type I IFN minimizes signs of genital disease. Loss of this control in IFNα/β$R^{-/-}$ mice causes rapidly worsening genital disease and permits virus dissemination from the periphery into the CNS (Fig. 5B). These findings attest to the importance of the type I IFN response in limiting virus infection and the significance of HSV-2 ICP34.5 in antagonizing it in vivo to promote viral replication and dissemination to the nervous system.

More than half of wt mice infected i.vag. with $\gamma34.5^{-/-}$ eventually succumbed to infection (Fig. 4A) despite the relatively low viral titers detected in the spinal cords of cohorts 6 d p.i. (Fig. 3C). This result caused us to question whether the type I IFN response delayed but did not prevent $\gamma34.5^{-/-}$ amplification in the nervous system. However, CNS tissues of wt mice infected i.vag. with $\gamma34.5^{-/-}$ that were moribund on day 13 p.i., did not contain detectable virus (data not shown). From these experiments it appears that $\gamma34.5^{-/-}$ is able to enter the spinal cord by or before day 6 p.i., but infection in the CNS is subsequently controlled and morbidity results from some other pathologic outcome of infection (Luker et al., 2003; Parr and Parr, 2003).

Intracranial infection bypasses the hurdles inherent in dissemination from a mucosal site to the CNS, and allows direct assessment of neurovirulence. The low levels of $\gamma34.5^{-/-}$ in the brain of wt but not IFNα/β$R^{-/-}$ mice inoculated i.c. underscore the critical role of ICP34.5 in surmounting the barrier posed by the type I IFN response to HSV-2 replication in the brain. This property is specific to ICP34.5 because another mutant, ΔTK^{-}, was attenuated for replication in the brain of wt mice but failed to recover the

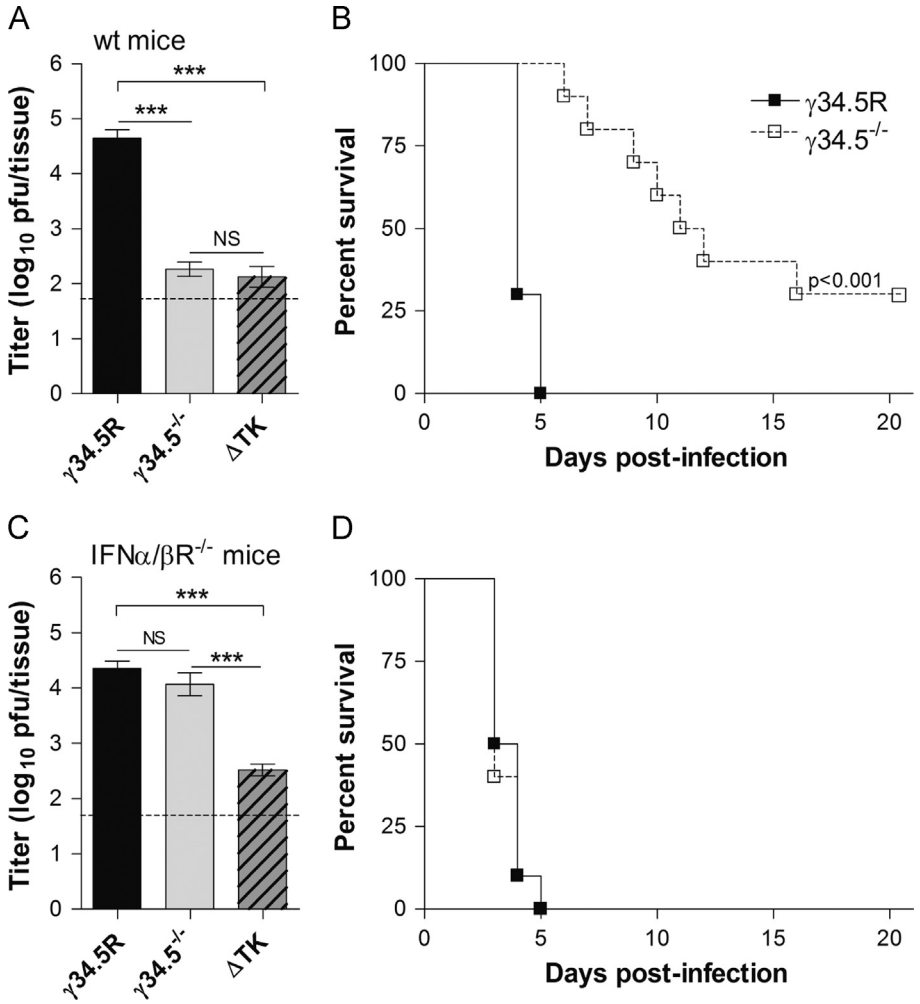

Fig. 6. $\gamma34.5^{-/-}$ replicates in the brains of IFNα/βR$^{-/-}$ mice and is highly lethal. (A) Wt mice and (B) IFNα/βR$^{-/-}$ mice were infected i.c. with 1×10^3 pfu/mouse of the indicated virus. Titer of virus in brain tissue was determined 24 h p.i. by plaque assay. Data represent the geometric mean+SEM of a total of 10 mice per group, compiled from three independent experiments. (C) Wt mice and (D) IFNα/βR$^{-/-}$ mice were infected i.c. with 1×10^3 pfu/mouse of the indicated virus and survival was monitored daily. Data represent the day of death of a total of 10 mice per group, compiled from two independent experiments. NS, no significance; ***, $p < 0.0001$. Dotted line represents limit of detection in the plaque assay.

capacity to replicate in the brain of IFNα/βR$^{-/-}$ mice. Increased replication of $\gamma34.5^{-/-}$ after i.c. inoculation of IFNα/βR$^{-/-}$ mice also directly correlated with increased mortality. These results indicate that the capacity of HSV-2 ICP34.5 to influence neurovirulence is directly related to its capacity to antagonize the type I IFN response. Interestingly, HSV-1 γ34.5 deletion mutants are non-lethal after corneal inoculation (Bolovan et al., 1994; Leib et al., 2000) and are markedly reduced in virulence after intracranial inoculation (Chou et al., 1990; Bolovan et al., 1994; Valyi-Nagy et al., 1994; Leib et al., 2000). The mortality we observed with $\gamma34.5^{-/-}$ suggests HSV-2 may have components in addition to ICP34.5 that contribute to its neurovirulence.

The inverted repeat regions of the HSV-2 genome where the two copies of γ34.5 reside are transcriptionally complex, suggesting mutations in γ34.5 could have pleotropic effects. The primary LAT is encoded on the strand opposite γ34.5 and is the source of several miRs: two miRs transcribed within LAT, miR-H4 and miR-H3, are complementary to sequences in the 5′ UTR and exon 1 of HSV-2 γ34.5, respectively (Tang et al., 2008, 2009), and both of these miRs reduce ICP34.5 expression during productive infection in culture (Tang et al., 2008, 2009). A third miR, miR-H24, is transcribed within LAT and is complementary to a sequence in the first exon of HSV-2 γ34.5 (Jurak et al., 2010). A fourth miR, miR-H19, arises from a sequence in the 5′ UTR of γ34.5 and is complementary to miR-H4 in the 5′ UTR of γ34.5 (Jurak et al., 2010). Importantly, the mutations we made to generate $\gamma34.5^{-/-}$ do not disrupt the pre-miRNAs or mature sequences for miR-H4, miR-H19 or miR-H24, and alter only a single base pairing in the miR-H3 pre-miRNA, although the impact of miRs suppressing ICP34.5 expression is expected to be minimal in a virus that does not synthesize ICP34.5. In addition, no evidence of internal initiation or alternative splicing of the HSV-2 γ34.5 transcript has been found (Korom et al., 2014). Thus, the phenotypes we observed in vitro and in vivo may be attributed to HSV-2 ICP34.5 because the specific mutations we introduced into both copies of γ34.5 selectively prevented ICP34.5 expression. Complete recovery of $\gamma34.5^{-/-}$ replication in IFNα/βR$^{-/-}$ MEFs and mice further attests that the mutations used to create $\gamma34.5^{-/-}$ do not disrupt regulatory features of the locus, and emphasize the importance of ICP34.5 in controlling the type I IFN response to amplify HSV-2 replication and pathogenesis.

Materials and methods

Cells and viruses

Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3% newborn calf serum

and 3% bovine growth serum (BGS), 2 mM L-glutamine and 1× penicillin–streptomycin (P/S) (Invitrogen). Primary 129, IFNα/βR$^{-/-}$, and PKR$^{-/-}$ MEFs were prepared from embryos at 15 to 18 d gestation and used through passage 4. MEFs were cultured in DMEM supplemented with 10% BGS and 1× P/S. MEFs (passage 1 to 4) were seeded into 24-well plates and allowed to divide until confluent (1×10^5 to 2×10^5 cells/well) or into 12-well plates (3×10^5 to 1×10^6 cells/well when confluent). Primary human foreskin fibroblasts (HFFs), derived from neonatal foreskins, were provided by David Sibley and were cultured in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% P/S. HFFs (passage 15–18) were seeded into 24-well plates and allowed to divide until confluent (1×10^5 to 2×10^5 cells/well).

All HSV-2 strains were in the wt strain 333 background (Duff and Rapp, 1971). To generate a γ34.5 deletion virus, a BamHI fragment of genomic DNA from HSV-2 strain 333 containing the γ34.5 gene was cloned into the XhoI and EcoRI sites in pBS-KS(+), creating plasmid pBS-34.5gDNA (Korom et al., 2014). The SanDI restriction site was utilized for insertion of a stop codon at amino acid 13 by means of a short oligo-linker containing a HpaI restriction site. Briefly, oligos (Fwd 5′-GTCCCCGTTAACGC-CGCCGGG-3′ and Rev 5′-GACCCGGCGGCGTTAACGGG-3′) were suspended in STE buffer (10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA) at 100 μM concentration. The two strands were mixed in equimolar amounts, heated to 95 °C for 5 min and allowed to gradually cool. The resulting double-stranded DNA fragment was added at 10 μM concentration to a ligation reaction also containing pBS-34.5 gDNA that had been digested with SanDI. The resulting plasmid, pBS-34.5-HpaI, was verified by restriction digest and sequencing. Two PCR fragments were generated from the pBS-34.5-HpaI plasmid to introduce an M35A mutation in the γ34.5 gene and add a KpnI restriction site. The first amplicon was generated using primers Fwd 5′-CTTAAGAGGGCCCGCAACAC-3′ and Rev-5′-CGCGGTACCCGAGTCGTACGCAGGGACCGCTTGGGAGT-CTGCGGTTGGGAGC-3′. The resulting three nucleotide substitutions caused an amino acid change from Met to Ala and added a unique KpnI site. The second amplicon was generated using primers Fwd 5′GACTCGGGTACCGCGGTCGAGAGCGCGCCGGCCGCG-TCCTCGCTC-3′ and Rev 5′- CCCGGGCTGCAGGAATTCATTAGCATAC-TAGGAAGCCCAGG-3′. The first PCR amplicon was digested with HpaI and KpnI, and the second amplicon was digested with KpnI and AgeI. A three-way ligation was performed with the amplicons and HpaI-AgeI digested pBS-34.5-HpaI. The resulting pBS-34.5$^{-/-}$ clones were screened by HpaI and KpnI digests and were verified by sequencing.

The mutated γ34.5 sequence in pBS-34.5$^{-/-}$ was incorporated into strain 333 viral DNA by homologous recombination after co-transfection using Amaxa Nucleofector and Kit V (Lonza). Isolated plaques were screened by PCR for replacement of both copies of γ34.5 and plaque-purified to homogeneity to create virus γ34.5$^{-/-}$. Restriction digests and sequencing verified the presence and location of the mutations. The γ34.5 rescue virus was isolated by co-transfection of full-length γ34.5$^{-/-}$ viral DNA with a linearized plasmid containing the wild-type γ34.5 sequence, followed by PCR screening of individual plaque isolates and plaque purification to homogeneity. Sequencing verified the replacement of both copies of γ34.5 in this virus, γ34.5R.

The γ34.5-null mutant of HSV-1 strain 17, Δγ34.5, was obtained from David Leib. Δγ34.5 contains a stop linker which causes truncation of ICP34.5 after amino acid 30 (Bolovan et al., 1994). Its marker rescue virus, Δγ34.5R, was isolated as previously described (Korom et al., 2013). HSV-2 ΔTK$^-$ is a TK-deficient mutant containing a 180 bp KpnI–KpnI deletion in the UL23 gene that eliminates TK activity (McDermott et al., 1984). Viruses were propagated on Vero cells and titers were determined by standard plaque assay (Knipe and Spang, 1982).

Western blot analyses

Vero cells were mock-infected or infected at MOI of 5. Cell monolayers were collected at 12 h p.i., lysed in 200 μl of lysis buffer, and stored at −20 °C. Frozen lysates were thawed and heated to 95 °C for 5 min, and 20 μl were loaded into a 14% SDS-PAGE gel. After electrophoresis, proteins were transferred to PVDF membranes which were blocked in Tris-buffered saline-Tween 20 (TBST) containing 5% nonfat dry milk and incubated with rabbit polyclonal antisera to HSV-2 ICP34.5 (Korom et al., 2014), anti-eIF2α (Santa Cruz Biotechnology), or anti-phospho eIF2α (Invitrogen). An anti-rabbit alkaline phosphatase-conjugated antibody (Promega) was used for detection. The primary and secondary antibody incubations were carried out in TBST. Bands were visualized using BCIP/NBT (Promega) according to the manufacturers' instructions.

In vitro infection and assessment of virus replication

Primary MEFs generated from 129 wt, IFNα/βR$^{-/-}$ and PKR$^{-/-}$ mice were seeded into 24-well plates. Confluent monolayers were infected with HSV-1 and HSV-2 strains at high MOI (10 pfu/cell) or low MOI (0.01 pfu/cell). At 1 h p.i. virus was removed and cells were gently washed with phosphate-buffered saline (PBS). Growth medium was added to each well for the remainder of the incubation. Infected cultures were collected by scraping at specific time points and stored at −80 °C until use. Immediately following sonication of samples, viral titers were determined by standard plaque assay.

In vitro IFNβ treatment and infection

Confluent MEFs were incubated with medium containing 100 or 1000 International Units/ml (IU/ml) of mouse IFNβ (PBL Interferon Source), or with medium only. After 24 h of IFNβ treatment, cells were gently washed twice with PBS and infected at high MOI (10 pfu/cell) with various viruses. At 1 h p.i. virus was removed, cells were gently washed with PBS, and DMEM supplemented with 10% BGS was added for the remainder of the incubation period. The contents of the well were collected 24 h p.i. and frozen at −80 °C. Virus titers were determined by standard plaque assay on Vero cells. Pre-treatment of HFFs followed the same procedure with the exceptions of recombinant human IFNβ (Prospec) and culture medium.

Animal studies

129 wt mice and IFNα/βR$^{-/-}$ mice (129 background) (Muller et al., 1994) were handled in strict accordance with good animal practice as defined by Institutional and Public Health Service guidelines, and with work approved by the Institutional Animal Care and Use Committee. The animals were housed and bred in the Saint Louis University School of Medicine Department of Comparative Medicine. Mice were treated subcutaneously with Depo-Provera (3 mg/mouse) at 5 weeks of age and again one day before i.vag. infection at 6 weeks of age. Groups of anesthetized mice were infected with 1×10^4 pfu/mouse of various viruses as previously described (Korom et al., 2008). The vaginas of mice were swabbed on days 1 through 5 p.i. with calcium alginate-tipped applicators. The swabs were placed in a vial containing 1 ml of PBS and stored at −80 °C until use. On days 1 through 6 p.i., mice were weighed and scored for genital disease using the scale: 0 no disease, 1 mild erythema, 2 moderate erythema and inflammation, 3 genital lesions. 6 d p.i., mice were euthanized and the brain, brainstem and spinal cord were dissected and placed into microfuge tubes containing 1 ml PBS and 1 mm glass beads.

To assess neurovirulence, groups of anesthetized mice were infected i.c in the right cortex with 1×10^3 pfu/mouse using a 29-gauge needle and 0.33 ml syringe (Terumo). Brain tissue was dissected at 24 h p.i. Tissues were disrupted using a Mini Beadbeater 8 (Prospec). Virus titers in vaginal swabs and homogenized tissues were determined by plaque assay. Survival following i.vag and i.c infections was monitored for 21 d p.i. and mice were sacrificed if moribund. In some cases CNS tissues were dissected 13 d p.i. for determination of virus titer.

Statistics

Statistical significance of differences in viral titers was determined by unpaired t test, in disease scores by the Kruskal–Wallis test, and in survival curves by the Kaplan–Meier test.

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References

Bolovan, C.A., Sawtell, N.M., Thompson, R.L., 1994. ICP34.5 mutants of herpes simplex virus type 1 strain 17syn+ are attenuated for neurovirulence in mice and for replication in confluent primary mouse embryo cell cultures. J. Virol. 68, 48–55.

Carr, D.J.J., Tomanek, L., 2006. Herpes simplex virus and the chemokines that mediate the inflammation. Curr. Top. Microbiol. Immunol. 303, 47–65.

Cheng, G., Brett, M.E., He, B., 2001a. Val193 and Phe195 of the gamma 1 34.5 protein of herpes simplex virus 1 are required for viral resistance to interferon-alpha/beta. Virology 290, 115–120.

Cheng, G., Gross, M., Brett, M.E., He, B., 2001b. AlaArg motif in the carboxyl terminus of the gamma(1)34.5 protein of herpes simplex virus type 1 is required for the formation of a high-molecular-weight complex that dephosphorylates eIF-2alpha. J. Virol. 75, 3666–3674.

Chou, J., Kern, E.R., Whitley, R.J., Roizman, B., 1990. Mapping of herpes simplex virus-1 neurovirulence to gamma 134.5, a gene nonessential for growth in culture. Science 250, 1262–1266.

Duerst, R.J., Morrison, L.A., 2004. Herpes simplex virus 2 virion host shutoff protein interferes with type I interferon production and responsiveness. Virology 322, 158–167.

Duerst, R.J., Morrison, L.A., 2007. Herpes simplex virus type 2-mediated disease is reduced in mice lacking RNase L. Virology 360, 322–328.

Duff, R., Rapp, F., 1971. Oncogenic transformation of hamster cells after exposure to herpes simplex virus type 2. *Nat. New Biol.* 233, 48–50.

Emeny, J.M., Morgan, M.J., 1979. Regulation of the interferon system: evidence that Vero cells have a genetic defect in interferon production. J. Gen. Virol. 43, 247–252.

Gill, N., Deacon, P.M., Lichty, B., Mossman, K.L., Ashkar, A.A., 2006. Induction of innate immunity against herpes simplex virus type 2 infection via local delivery of Toll-like receptor ligands correlates with beta interferon production. J. Virol. 80, 9943–9950.

He, B., Gross, M., Roizman, B., 1997. The gamma (1) 34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1 alpha to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA 94, 843–848.

He, B., Gross, M., Roizman, B., 1998. The gamma134.5 protein of herpes simplex virus 1 has the structural and functional attributes of a protein phosphatase 1 regulatory subunit and is present in a high molecular weight complex with the enzyme in infected cells. J. Biol. Chem. 273, 20737–20743.

Jancel, T., Penzak, S.R., 2009. Antiviral therapy in patients with hematologic malignancies, transplantation, and aplastic anemia. Semin. Hematol. 46, 230–247.

Jurak, I., Kramer, M.F., Mellor, J.C., van Lint, A.L., Roth, F.P., Knipe, D.M., Coen, D.M., 2010. Numerous conserved and divergent microRNAs expressed by herpes simplex viruses 1 and 2. J. Virol. 84, 4659–4672.

Kimberlin, D.W., Whitley, R.J., 2005. Neonatal herpes: what have we learned. Semin. Pediatr. Infect. Dis. 16, 7–16.

Knipe, D.M., Spang, A.E., 1982. Definition of a series of stages in the association of two herpesviral proteins with the cell nucleus. J. Virol. 43, 314–324.

Korom, M., Wylie, K.M., Morrison, L.A., 2008. Selective ablation of virion host shutoff protein RNase activity attenuates herpes simplex virus 2 in mice. J. Virol. 82, 3642–3653.

Korom, M., Wylie, K.M., Wang, H., Davis, K.L., Sangabathula, M.S., Delassus, G.S., Morrison, L.A., 2013. A proautophagic antiviral role for the cellular prion protein identified by infection with a herpes simplex virus 1 ICP34.5 mutant. J. Virol. 87, 5882–5894.

Korom, M., Davis, K.L., Morrison, L.A., 2014. Up to four distinct polypeptides are produced from the γ34.5 open reading frame of herpes simplex virus 2. J. Virol. 88, 11284–11296.

Leib, D.A., Harrison, T.E., Laslo, K.M., Machalek, M.A., Moorman, N.J., Virgin, H.W., 1999. Interferons regulate the phenotype of wild-type and mutant herpes simplex viruses in vivo. J. Exp. Med. 189, 663–672.

Leib, D.A., Machalek, M.A., Williams, B.R., Silverman, R.H., Virgin, H.W., 2000. Specific phenotypic restoration of an attenuated virus by knockout of a host resistance gene. Proc. Natl. Acad. Sci. USA 97, 6097–6101.

Li, Y., Zhang, C., Chen, X., Yu, J., Wang, Y., Yang, Y., Du, M., Jin, H., Ma, Y., He, B., Cao, Y., 2011. ICP34.5 protein of herpes simplex virus facilitates the initiation of protein translation by bridging eukaryotic initiation factor 2alpha (eIF2alpha) and protein phosphatase 1. J. Biol. Chem. 286, 24785–24792.

Looker, K.J., Garnett, G.P., Schmid, G.P., 2008. An estimate of the global prevalence and incidence of herpes simplex virus type 2 infection. Bull. World Health Organ. 86, 805–812.

Luker, G.D., Prior, J.L., Song, J., Pica, C.M., Leib, D.A., 2003. Bioluminescence imaging reveals systemic dissemination of herpes simplex virus type 1 in the absence of interferon receptors. J. Virol. 77, 11082–11093.

Ma, Y., Jin, H., Valyi-Nagy, T., Cao, Y., Yan, Z., He, B., 2012. Inhibition of TANK binding kinase 1 by herpes simplex virus 1 facilitates productive infection. J. Virol. 86, 2188–2196.

McDermott, M.R., Smiley, J.R., Leslie, P., Brais, J., Rudzroga, H.E., Bienenstock, J., 1984. Immunity in the female genital tract after intravaginal vaccination of mice with an attenuated strain of herpes simplex virus type 2. J. Virol. 51, 747–753.

McGeoch, D.J., Cunningham, C., McIntyre, G., Dolan, A., 1991. Comparative sequence analysis of the long repeat regions and adjoining parts of the long unique regions in the genomes of herpes simplex viruses types 1 and 2. J. Gen. Virol. 72 (Pt 12), 3057–3075.

Mommeja-Marin, H., Lafaurie, M., Scieux, C., Galicier, L., Oksenhendler, E., Molina, J. M., 2003. Herpes simplex virus type 2 as a cause of severe meningitis in immunocompromised adults. Clin. Infect. Dis. 37, 1527–1533.

Mosca, J.D., Pitha, P.M., 1986. Transcriptional and posttranscriptional regulation of exogenous human beta interferon gene in simian cells defective in interferon synthesis. Mol. Cell. Biol. 6, 2279–2283.

Muller, U., Steinhoff, U., Reis, L.F., Hemmi, S., Pavlovic, J., Zinkernagel, R.M., Aguet, M., 1994. Functional role of type I and type II interferons in antiviral defense. Science 264, 1918–1921.

Mulvey, M., Poppers, J., Ladd, A., Mohr, I., 1999. A herpesvirus ribosome-associated, RNA-binding protein confers a growth advantage upon mutants deficient in a GADD34-related function. J. Virol. 73, 3375–3385.

Mulvey, M., Poppers, J., Sternberg, D., Mohr, I., 2003. Regulation of eIF2alpha phosphorylation by different functions that act during discrete phases in the herpes simplex virus type 1 life cycle. J. Virol. 77, 10917–10928.

Murphy, J.A., Duerst, R.J., Smith, T.J., Morrison, L.A., 2003. Herpes simplex virus type 2 virion host shutoff protein regulates alpha/beta interferon but not adaptive immune responses during primary infection in vivo. J. Virol. 77, 9337–9345.

Orvedahl, A., Alexander, D., Talloczy, Z., Sun, Q., Wei, Y., Zhang, W., Burns, D., Leib, D. A., Levine, B., 2007. HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. Cell Host Microbe 1, 23–35.

Parr, M.B., Kepple, L., McDermott, M.R., Drew, M.D., Bozzola, J.J., Parr, E.L., 1994. A mouse model for studies of mucosal immunity to vaginal infection by herpes simplex virus type 2. Lab. Investig. 70, 369–380.

Parr, M.B., Parr, E.L., 2003. Intravaginal administration of herpes simplex virus type 2 to mice leads to infection of several neural and extraneural sites. J. Neurovirol. 9, 594–602.

Pinninti, S.G., Kimberlin, D.W., 2013. Maternal and neonatal herpes simplex virus infections. Am. J. Perinatol. 30, 113–119.

Taha, M.Y., Clements, G.B., Brown, S.M., 1989a. A variant of herpes simplex virus type 2 strain HG52 with a 1.5 kb deletion in RL between 0 to 0.02 and 0.81 to 0.83 map units is non-neurovirulent for mice. J. Gen. Virol. 70 (Pt 3), 705–716.

Taha, M.Y., Clements, G.B., Brown, S.M., 1989b. The herpes simplex virus type 2 (HG52) variant JH2604 has a 1488 bp deletion which eliminates neurovirulence in mice. J. Gen. Virol. 70, 3073–3078.

Talloczy, Z., Jiang, W., Virgin 4th, H.W., Leib, D.A., Scheuner, D., Kaufman, R.J., Eskelinen, E.L., Levine, B., 2002. Regulation of starvation- and virus-induced autophagy by the eIF2alpha kinase signaling pathway. Proc. Natl. Acad. Sci. USA 99, 190–195.

Tang, S., Bertke, A.S., Patel, A., Wang, K., Cohen, J.I., Krause, P.R., 2008. An acutely and latently expressed herpes simplex virus 2 viral microRNA inhibits expression of ICP34.5, a viral neurovirulence factor. Proc. Natl. Acad. Sci. USA 105, 10931–10936.

Tang, S., Guo, N., Patel, A., Krause, P.R., 2013. Herpes simplex virus 2 expresses a novel form of ICP34.5, a major viral neurovirulence factor, through regulated alternative splicing. J. Virol. 87, 5820–5830.

Tang, S., Patel, A., Krause, P.R., 2009. Novel less-abundant viral microRNAs encoded by herpes simplex virus 2 latency-associated transcript and their roles in regulating ICP34.5 and ICP0 mRNAs. J. Virol. 83, 1433–1442.

Valyi-Nagy, T., Fareed, M.U., O'Keefe, J.S., Gesser, R.M., MacLean, A.R., Brown, S.M., Spivack, J.G., Fraser, N.W., 1994. The herpes simplex virus type 1 strain 17+ gamma 34.5 deletion mutant 1716 is avirulent in SCID mice. J. Gen. Virol. 75 (Pt 8), 2059–2063.

Verpooten, D., Feng, Z., Valyi-Nagy, T., Ma, Y., Jin, H., Yan, Z., Zhang, C., Cao, Y., He, B., 2009. Dephosphorylation of eIF2alpha mediated by the gamma 134.5 protein of herpes simplex virus 1 facilitates viral neuroinvasion. J. Virol. 83, 12626–12630.

Verpooten, D., Ma, Y., Hou, S., Yan, Z., He, B., 2009. Control of TANK-binding kinase 1-mediated signaling by the gamma(1)34.5 protein of herpes simplex virus 1. J. Biol. Chem. 284, 1097–1105.

Wylie, K.M., Schrimpf, J.E., Morrison, L.A., 2009. Increased eIF2alpha phosphorylation attenuates replication of herpes simplex virus 2 vhs mutants in mouse embryonic fibroblasts and correlates with reduced accumulation of the PKR antagonist ICP34.5. J. Virol. 83, 9151–9162.